Artificial reagents for factor VII and factor X

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Artificial Reagents for Factor VII and Factor X, a Computer Programme for Obtaining Reference Tables for One-Stage Determinations in the Extrinsic System

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In the course of an investigation on the separation of the coagulation factors II, VII, IX and X (8), the need was felt for large quantities of reagents to assess the factors VII and X separately. Because congenitally deficient plasmas are scarce, we developed a procedure to obtain from normal plasmas preparations deficient in factor VII or factor X, starting in principle from the methods for preparing factor VII deficient plasma as described by Lechner and Deutsch (6) and Casillas and Simonetti (2).

Materials and Methods

Coagulation tests

0.1 ml reagent, 0.1 ml thromboplastin (prepared according to Owren and Aas (7), Quick time with normal pool plasma 13–15 sec), and 0.1 ml sample are incubated at 37.0° C for 30 ± 1 sec. The reaction is then started by addition of 0.1 ml $CaCl_2$ solution 30 mM, preheated to the same temperature. The addition is made by means of an Eppendorf pipette.

Reference values

Reference values are recorded by assessing the coagulation times obtained with known dilutions of normal plasma in veronal acetate buffer pH 7.4.

Factor V and II reagents

These reagents were prepared according to Koller et al. (5) (factor II) and Kahn and Hemker (4) (factor V).

Normal pool plasma

98 parts of freshly drawn blood are mixed with 2 parts 0.55 M sodium citrate in a plastic container. The cells are sedimented by 15 min centrifugation at 1250 g. The plasma is centrifuged for 30 min at 20.000 g to carry down the platelets. The platelet free plasma obtained in this way from at least 30 donors, sex ratio 1:1, of a mean age of 30 (21–47) is mixed and stored in plastic at –20° C.

Veronal acetate buffer

0.0286 M sodium acetate
0.0286 M sodium barbiturate
0.1164 M NaCl
0.0200 M HCl

The pH is 7.4.

Preparation of the Reagents

Factor VII reagent

This is prepared according to a modification of the method of Lechner and Deutsch (6). 5 g of DEAE-cellulose (SIGMA) are equilibrated with a solution 13 mM in sodium citrate and 0.135 M in sodium chloride, and are poured in a Pharmacia K 25/45 column. 150 ml ACD plasma, diluted

with 100 ml of the sodium citrate sodium chloride solution are passed through the column followed by a solution 6.5 M in sodium citrate and 0.1425 M in sodium chloride. The column is washed until no 280 nm · absorbing material can be detected in the effluent. The adsorbed proteins then are eluted with 0.02 M sodium potassium phosphate buffer pH 7.0, containing 1 M sodium chloride. The eluate after dialysis against Veronal acetate buffer is added to barium sulphate adsorbed oxalated bovine plasma in such an amount that after dilution with Veronal acetate buffer to twice the volume of the barium sulphate adsorbed plasma, the factor II activity is 30–40% of normal pool plasma. The reagent gives results that show a perfect correlation with those obtained with congenitally factor VII deficient plasma (Fig. 1).

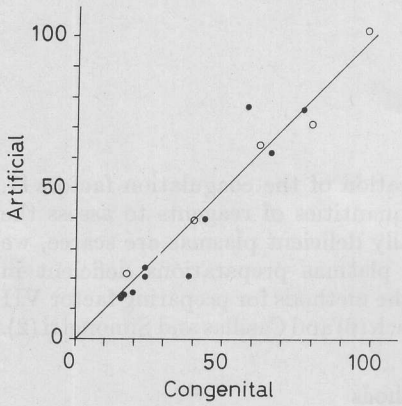

Fig. 1.

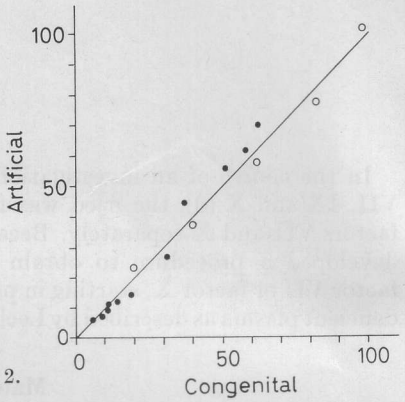

Fig. 2.

Fig. 1. *Comparison of the factor VII reagent and a congenitally deficient plasma.* The concentration (% of normal plasma) estimated with the reagent described is plotted (ordinate) against the concentration estimated with the congenitally deficient plasma used as a reagent (abcissa). ●: pooled plasmas of patients anticoagulated at different levels, ○: mixtures of normal plasma and $Al(OH)_3$ adsorbed plasma.

Fig. 2. *Comparison of the factor X reagent and a congenitally deficient plasma.* The concentration (% of normal plasma) estimated with the reagent described is plotted (ordinate) against the concentration estimated with the congenitally deficient plasma used as a reagent (abcissa). ●: pooled plasmas of patients anticoagulated at different levels, ○: mixtures of normal plasma and $Al(OH)_3$ adsorbed plasma.

Factor X reagent

The plasma that leaves the DEAE-cellulose column used for the preparation of a factor VII reagent, contains factor VII and only a small amount of factor X. The plasma is adsorbed with 5 g of aluminium hydroxide per litre of plasma. The adsorbed proteins are eluted with one-tenth plasma volume 0.25 M sodium potassium phosphate buffer pH 8.0. A saturated solution of ammonium sulphate is then added so as to obtain 40% saturation. This procedure precipitates part of the proteins, they are centrifuged down (10 min 10.000 g) and discarded.

From the supernatant factor VII and a small amount of factor X are precipitated by raising the ammonium sulphate concentration to 65% saturation. The precipitate is collected as above, and dissolved in a few ml of water and dialyzed against 0.02 M sodium potassium phosphate pH 6.8 0.1 M sodium chloride. After dialysis the solution is applied to a DEAE-Sephadex column (1,5 × 20 cm) equilibrated with the same buffer. The adsorbed proteins are eluted with a linear gradient of sodium chloride from 0.1 to 1 M in 0.02 M phosphate pH 6.8.

Factor VII is eluted well before factor X. The purified factor VII preparation is added to Seiz-filtered oxalated bovine plasma, prepared according to Bachmann et al. (1). The factor VII activity in the reagent is made 30% of that in normal pool plasma. The results obtained with this reagent are in complete agreement with those given by a congenitally factor X deficient plasma (Fig. 2).

Properties of the reagents

The mean coagulation factor concentrations of the reagents are shown in Table 1. The way in which the residual amount of factors X and VII is estimated is described below.

In Table 2 the buffer times and the coagulation times with a sample of 1% and 10% of normal plasma are given.

Table 1. Factor concentration in the reagents.

Coagulation factor	Factor VII	Factor X
Fibrinogen (mg/ml)	1.23 (1.05–1.40)	1.32 (1.27–1.37)
Factor II (%)	31 (24–44)	28 (24–32)
Factor V (%)	25 (16–30)	43 (31–55)
Factor VII (%)	0.8 (1.1–0.3)	35 (33–36)
Factor X (%)	37 (24–54)	1.1 (0.7–1.7)

The figures give the means and limits of the values obtained in 7 (factor VII) and 8 (factor X) batches of reagent.

Table 2. Coagulation times obtained with the reagents.

Concentration normal plasma in sample (%)	Coagulation time (sec)	
	Factor VII	Factor X
10	25.6 (21.8–29.6)	26.0 (24.0–28.9)
1	47.6 (42.3–64.0)	53.5 (45.9–62.7)
0	80.9 (60.8–142.1)	71.8 (64.6–89.3)

The figures give the means and limits of the coagulation obtained in 7 (factor VII) and 8 (factor X) batches of reagent.

Reference Curves

With the reagents described the usual type of double logarithmic reference curves can be made. Figs. 3 and 4 give representative examples. These graphs are indistinguishable from straight lines when a limited range of concentration, e. g. 1–10%, is used. These graphs therefore cannot be used beyond the region in which it has been shown experimentally that the linear relationship does hold.

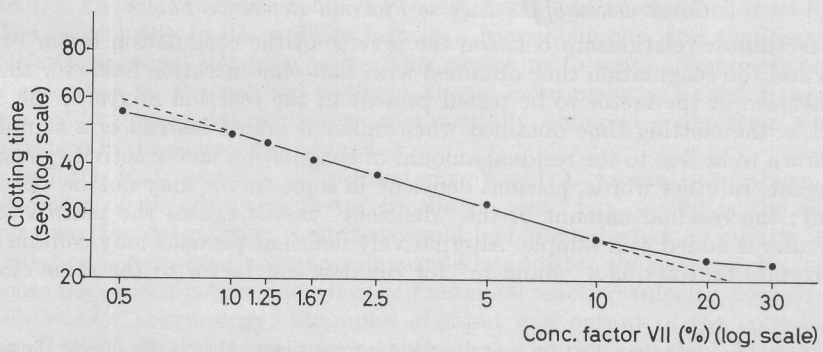

Fig. 3. A log-log reference curve for the factor VII reagent.

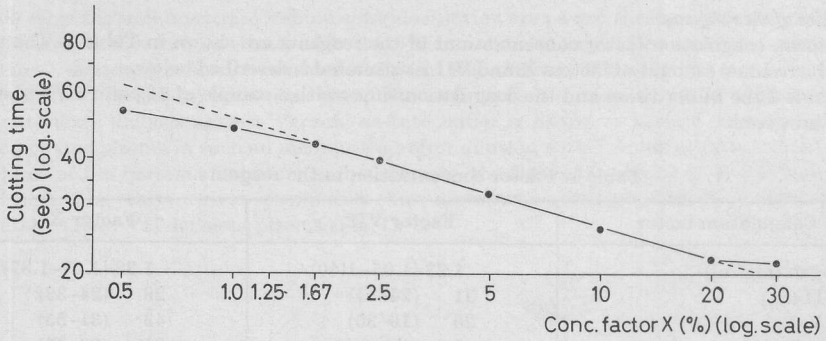

Fig. 4. A log-log reference curve for the factor X reagent.

This is not particular to the X and VII reagent described here. Double logarithmic graphs obtained over a wide range of concentration will be S-shaped with *any* type of reagent, this is often not sufficiently realized. On the other hand for estimations in the extrinsic system there *does* exist a relationship which has been shown to hold for the complete range of values for which it has been able to put it to test. This is the rectilinear relationship between the coagulation time and the inverse of the concentration[1]) of the factor tested (i. e. the rate limiting factor, i. e. factor VII in a VII reagent etc.). The theoretical basis for the relationship can be explained from the reaction mechanism of the extrinsic system (3). We will call this relationship the inverse relationship (and the plot obtained with it the inverse plot) as opposed to the double logarithmic relationship (c. q. double logarithmic plot).

A relationship between clotting time and concentration that has been proven to be valid over the complete range of concentration has two advantages:

1. extrapolation beyond the range of the concentration used to obtain the reference values is valid, and
2. deviations from rectilinearity can be interpreted as random experimental scatter, so that standard statistical procedures can be used to overcome their effect.

In the double logarithmic plot deviations of rectilinearity may be systematic, and therefore give rise to unduely large estimation of the experimental error when they are treated as random errors.

Construction of the Inverse Plot and Reference Tables

The rectilinear relationship between the inverse of the coagulation factor concentration and the coagulation time obtained with that concentration holds for the final concentration of the factor to be tested present in the reaction mixture; the buffer value, i. e. the clotting time obtained when buffer is added instead of a sample has been shown to be due to the residual amount of coagulation factor activity present in the reagent. In other words, plasmas deficient in some factor may not be *completely* deficient; the residual amount of the "deficient" factor causes the plasma to clot when buffer is added as a sample. Alternatively deficient plasmas may contain other (pro)enzymes that act as a "stand in" for the deficient factor to the same effect of

1) It should be clear that the *total* concentration is meant here; that is the sum of the amount added and the amount present in the reagent.

residual activity. This view has been the hypothesis at the basis of a series of kinetical experiments. It has been shown to be compatible with the outcome of these experiments (3) and therefore will be maintained as the basis of the following discussion.

In a test for a specific factor the total concentration of that factor present in the reaction mixture is rate-limiting.

The final concentration (F) in the test is the sum of the concentration added with the sample (C) and the concentration added with the reagent (L). So $F = C + L$. In obtaining reference values, C is known, but L is not. The inverse relation holds between the final concentration and the coagulation time (t). In other symbols

$$t_c = a.\frac{1}{F} + b \tag{1}$$

or

$$t_c = a\frac{1}{L+C} + b \tag{2}$$

At a concentration of C added, we call the clotting time obtained t_c. When buffer is added the coagulation time (i. e. the buffer value t_l) is due to L only, as $C = 0$ then. This is a special case of formula (2).

$$t_l = a\frac{1}{L} + b \tag{3}$$

Rearranging formulas (1) and (2) according to the rules of basic algebra gives

$$t_c = L\frac{t_l - t_c}{C} + b \tag{4}$$

or

$$t_c = L \cdot V_c + b \tag{5}$$

where V_c $(= \frac{t_l - t_c}{C})$ is a new variable consisting of known (C) and experimentally obtainable (t_l, t_c) values. It is easily seen that the plot of t_c versus V_c is a straight line with the direction coefficient L. L, the concentration of the rate limiting factor in the reagent can therefore be read from the graph, in which t_c is plotted as a function of V_c. In this way the residual concentration of factor VII and X in the reagents described were estimated (Table 1).

Once L is known, the relationship between t_c and C can be obtained from formula (2). This relationship in its explicite form is a hyperbolic one, and therefore is less suitable as a practical reference curve. This caused us to write a computer program which according to the method outlined above, determines a, L, and b, and then prints a table from which for each experimentally obtained coagulation time the concentration in the sample can be read.

Values for L, t_{min} and K_m are printed out as well. L, as explained above, is the concentration of the deficient factor in the reagent; t_{min} $(= b)$ is the minimal clotting time (the coagulation time that would be obtained when an infinite amount of coagulation factor under consideration would be added to the reagent); K_m $(= a : b)$ represents the concentration that gives half maximal reaction velocity, comparable to K_m in standard enzymology. Examples of input and output of the computer are given in Table 3. The program can be obtained at cost price in Algol, Fortran IV or FOCAL (PDP 8 or PDP 12) from the authors.

Table 3. *Input and output of the FOCAL program.*

In FOCAL the program is in a conversational form. After the GO command, the machine prints the input section, in which the underlined data have to be typed in by hand. When only the factor concentration in the reagent, the minimal time and the K_m are wanted, the last question is answered with *no*, and the program comes to an end. The reference table goes from the first full number of seconds above the minimum time to the first full number of seconds above the longest experimental coagulation time given in the input. Only even first decimals are given, odds can be interpolated.

INPUT

```
GO
REAGENT-FACTOR      :VII                DATE: 13 :Ø5 :71
BATCH NO            :7Ø8
THROMBOPLASTIN NO   :1921
NORMAL PLASMA NO    :15Ø9
CA-CHLORIDE       1:3Ø MOLAIR
        BUFFER TIME:66.8 SEC.
    NUMBER OF POINTS:8
-------------------------------------
CONCENTRATION                COAGULATION-TIME
    :2Ø.ØØ      %                :22.3    SEC.
    :1Ø.ØØ                       :24.9
    : 5.ØØ                       :29.2
    : 2.5Ø                       :32.6
    : 1.67                       :37.8
    : 1.25                       :41.4
    : 1.ØØ                       :45.4
    : Ø.5Ø                       :53.3
```

OUTPUT

```
FACTOR IN REAGENT                = 1.18 %
MINIMAL TIME                     = 19.37 SEC.
HALF-TIME CONCENTRATION (KM) =  2.85 %

DO YOU WANT A REFERENCE-TABLE  YES OR NO ? : YES
```

* COAGULATION TIME (SEC.)	CONCENTRATION (%)				
*	.Ø	.2	.4	.6	.8
* = 21	= 32.73	= 29.Ø2	= 26.Ø5	= 23.61	= 21.57
* = 22	= 19.84	= 18.35	= 17.Ø6	= 15.93	= 14.93
* = 23	= 14.Ø5	= 13.25	= 12.53	= 11.89	= 11.3Ø
* = 24	= 1Ø.76	= 1Ø.26	= 9.81	= 9.39	= 9.ØØ
* = 25	= 8.64	= 8.3Ø	= 7.99	= 7.69	= 7.42
* = 26	= 7.16	= 6.91	= 6.68	= 6.47	= 6.26
* = 27	= 6.Ø6	= 5.88	= 5.7Ø	= 5.54	= 5.38
* = 28	= 5.23	= 5.Ø8	= 4.94	= 4.81	= 4.68
* = 29	= 4.56	= 4.44	= 4.33	= 4.22	= 4.12
* = 3Ø	= 4.Ø2	= 3.92	= 3.83	= 3.74	= 3.66
* = 31	= 3.57	= 3.49	= 3.42	= 3.34	= 3.27
* = 32	= 3.2Ø	= 3.13	= 3.Ø6	= 3.ØØ	= 2.94
* = 33	= 2.88	= 2.82			
			ETC.		
				= Ø.31	= Ø.3Ø
* = 57	= Ø.29	= Ø.28	= Ø.28	= Ø.27	= Ø.26
* = 58	= Ø.25	= Ø.25	= Ø.24	= Ø.23	= Ø.22
* = 59	= Ø.22	= Ø.21	= Ø.2Ø	= Ø.2Ø	= Ø.19

Summary

A method is described to prepare artificial reagents to test the factors VII and X individually.

A procedure is given to obtain reference tables suitable for the use of these reagents as well as for any other type of specific one-stage in the extrinsic system by means of a computer. The programme is obtainable in Algol, Fortran or Focal.

Résumé

On décrit une méthode pour préparer des réactif artificiels pour la détermination séparée des facteurs VII et X. On indique un procédé pour l'obtention de tables de références pour l'utilisation de ces réactifs ou de tout autre type de méthode en un temps spécifique dans le système extrinsèque et à l'aide d'un ordinateur. On peut obtenir le programme en Algol, Fortran ou Focal.

Zusammenfassung

Es wird eine Methode zur Herstellung künstlicher Reagenzien für die getrennte Bestimmung der Faktoren VII und X beschrieben.

Es wird ein Vorgehen mitgeteilt, das ermöglicht, Referenztabellen zu erhalten, welche für die Verwendung mit diesen Reagenzien, aber auch für jede andere Art des spezifischen Einstufentestes im exogenen System mit Hilfe eines Computers geeignet sind. Das Programm ist verfügbar in Algol, Fortran und Focal.

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